stantia nigra, indicating that ε-SG is expressed in dopaminergic neurons (Fig. 7M–O). Neither Esg-C1 nor Esg-C2 antibody was available for immunocytochemical study.

3.5. Subcellular localization of ε-SG isoforms in the mouse brain

We examined the expression pattern of ε-SG1 and ε-SG2 in various subcellular compartments of mouse brain to elucidate their roles in the central nervous system.

Fig. 8 shows the brain subcellular fractions prepared according to the procedure of Huttner et al. [18]. We first examined the distribution of several neural membrane markers, such as a marker for synaptic vesicles, synaptophysin; one for pre-synaptic membranes, SNAP-25; and one for post-synaptic membranes, PSD-95. Synaptophysin was remarkably enriched in the LP2 fraction but not in LP1 and PSD fractions, whereas PSD-95 was enriched in LP1 and PSD fractions but not in the LP2 fraction. These results were consistent with previous reports [12]. On the other hand, SNAP-25 was found in LP1 as well as the LP2 fraction, but not found in the PSD fraction. We then examined the expression of ε-SG1 and ε-SG2. ε-SG1 was mainly present in P2 and P3 fractions, but more concentrated in LP1 than in the LP2 fraction. ε-SG2 was also concentrated in P2 and P3 fractions, but distributed equally in LP1 and LP2 fractions. Neither isoform was enriched but certainly existed in the PSD fraction.

We further analyzed the expression of ε-SG isoforms in the brain capillary endothelial cell fraction purified with BS-1 lectin beads [9] (Fig. 8C). In the isolated endothelial cells, we were able to rule out the expression of a neuronal marker

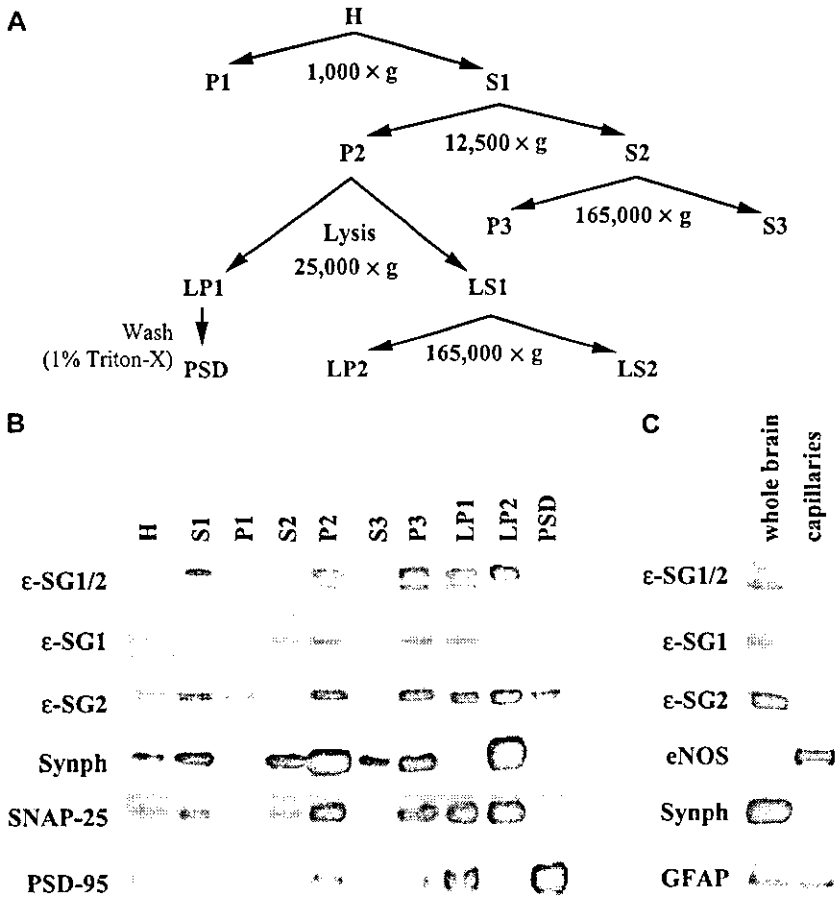

Fig. 8. Subcellular localization of ε-SG1 and ε-SG2 in adult mouse brain. (A) Schematic of the biochemical fractionation. The subcellular fractionation of mouse brain was carried out according to the procedure of Huttner et al. [18]. Fractions are as follows: H, total brain homogenates; P1, nuclei and large debris; P2, a crude synaptosomal fraction; P3, a light membrane/microsome-enriched fraction; LP1, a synaptosomal membrane fraction; LP2, a synaptic vesicle-enriched fraction. Each of the supernatants is designated S1, S2, or S3. A post-synaptic density fraction (PSD) was prepared from LP1 according to the method of Phillips et al [44]. (B) Detection of ε-SG isoforms in subcellular fractions from adult mouse brain. The isolated biochemical fractions were separated by SDS-PAGE (9% polyacrylamide gel) and then blotted with the antibodies, Esg-C1 (ε-SG1), Esg-C2 (ε-SG2), or Esg-Cyt (ε-SG1/2). Subcellular compartments were identified by detection of resident marker proteins with the corresponding antibodies. These markers are synaptophysin (Synph) for synaptic vesicles, SNAP-25 for pre-synaptic membranes, and PSD-95 for post-synaptic membranes. (C) Detection of ε-SG1 in brain capillaries. The capillary endothelial cells were isolated from the mouse brain by BS-1 lectin-beads [9], and the expression of ε-SG1 and ε-SG2 in the cells was analyzed by immunoblotting. Ten micrograms of proteins of mouse whole brain and the isolated capillaries were used for the analysis. eNOS, endothelial nitric oxide synthase, is a marker for endothelial cells.

(synaptophysin), but did detect an astrocyte marker (GFAP), indicating a close association of astrocytes with capillary endothelial cells. Immunoblotting using Esg-C1 and Esg-C2 antibodies clearly found ε-SG1, but not ε-SG2, in the cells. This result revealed that ε-SG1 is predominantly expressed in capillary endothelial cells and astrocytes.

4. Discussion

Mutations in the *ε-SG* gene (*SGCE*) cause M-D, indicating the functional importance of ε-SG in the central nervous system [51]. Despite much work on *SGCE* mutations in M-D families, very little is known about the localization and function of the protein product of *SGCE* in the central nervous system. In the present study, we found two isoforms of ε-SG in the mouse brain and investigated their distribution and localization.

We have shown the expression of two ε-SG isoforms in the mouse brain. One is identical to the ε-SG (ε-SG8$^+$/11b$^-$) that was initially discovered by cDNA cloning of mouse lung [14], and the other is a novel isoform excluding exon 8 and including exon 11b (ε-SG8$^-$/11b$^+$). We propose to designate the former ε-SG1 and the latter ε-SG2. The same results were obtained from human brain on mRNA and protein levels. In addition, ε-SG8$^-$/11b$^-$ type transcripts were markedly expressed in human brain (data not shown). This finding suggested that ε-SG2 plays some specific role in the mammalian brain.

Previous immunocytochemical studies showed ε-SG expression in a variety of cell types, i.e., striated and smooth muscles, capillary blood vessels, Schwann cells in peripheral nerves, alveoli and bronchioles in lung, and glomerular mesangium in kidney [14,20]. The present study showed that ε-SGs are widely distributed throughout the brain and that they are expressed in neuronal and non-neuronal cells including capillary endothelial cells and astrocytes. Immunolabeling of whole mouse brain sections revealed that the ε-SG expression was most marked in the neuronal cells within the olfactory bulb, hippocampus, cerebral cortex, pons, and cerebellar cortex. In almost all cases, ε-SG around cell bodies was more remarkable than that of fibrous structures.

Subcellular fractionation of brain homogenate suggested differential localization of ε-SG1 and ε-SG2 in synaptosomal membranes. We speculate that the two ε-SG isoforms, ε-SG1 and ε-SG2, play different roles at synapses in neurons, and this difference may relate to the structural difference of ε-SGs at their cytoplasmic domains (Fig. 1). Sequence analysis of this domain using the BLAST program (blastp) did not suggest any candidate gene, but a consensus sequence (Arg-Lys-Leu-Thr) for a phosphorylation site of cAMP- and cGMP-dependent protein kinase is present in the ε-SG2 C-terminal. To clarify the roles of ε-SG isoforms in the brain, it is important to search out proteins that interact with their unique cytoplasmic domains.

SGs (α, β, γ, and δ) are thought to function only when they form a subcomplex (SGC) within the dystrophin-DAP complex. ε-SG1 also has been shown to participate in forming SGCs in the smooth muscle and peripheral nervous system [20,47]. Therefore, the question arises whether the ε-SGs play a role as a constituent of the DAP complex that anchors dystrophin in the central nervous system or not. Several different promoters, scattered throughout the *dystrophin* gene, drive the tissue-specific expression of full-length dystrophins (427 kDa) and various short dystrophin isofroms including dystroglycan-binding sites, i.e., Dp260, Dp140, Dp116, and Dp71, in reference to their respective molecular sizes [5,11,17,27,30,35]. Among these dystrophins, full-length dystrophin (Dp427), Dp140, and Dp71 are expressed in the central nervous system. Dp427 is found almost exclusively in neurons in the cerebral cortex and cerebellar cortex, and localized along the plasma membrane of their perikaryons and the proximal dendrites [29,37]. It is also expressed in the hippocampal pyramidal cell layer but not in granule cells of the dentate gyrus. Within the cerebellar cortex, Dp427 is found in Purkinje cells but not in granule cells, Golgi cells, and basket cells. On the other hand, Dp140 is found in astrocytic processes throughout the neuropil, along penetrating microvasculature, and on the cells ensheathing olfactory neurons [30]. The major dystrophin isoform in the brain, Dp71, is expressed in the cerebral cortex, granule cells of the hippocampal dentate gyrus, olfactory bulb, and pituitary gland [34]. These brain dystrophins have been shown to form several types of complex with some dystrophin-accociated proteins, i.e., dystroglycans, syntrophins, and dystrobrevins [8,37]. However, to date, no dystrophin-DAP complex including an SGC has ever been found in the brain, although the expression patterns of the Dp427, Dp140, and Dp71 are totally overlapped with those of ε-SGs except for the neurons in the substantia nigra (Figs. 6 and 7). Besides ε-SGs, faint expression of β-, γ-, and δ-SG were found in whole brain materials at the transcriptional level [4,31,39,41]. We preliminarily tried to detect the SGs in the brain subcellular fractions containing ε-SGs by immunoblot, but found no evidence of their expression at the protein level (data not shown). In the central nervous system, especially in dorpaminergic neurons, the ε-SGs do not seem to form SGCs as a subcomplex of the dystrophin-DAP complex, but it is possible that the brain ε-SGs associate with other kinds of membrane proteins and/or cytoskeletal proteins in neuronal cells.

M-D is a movement disorder clinically characterized by myoclonus combined with dystonia. These abnormal movements can be caused by the pharmacological interference in dopaminergic and serotonergic neurotransmission. Indeed, dopa-responsive dystonia is caused by mutation in genes encoding enzymes of dopamine biosynthesis [19,24], and the transcript for *DYT1*, a gene responsible for early-onset torsion dystonia, is shown to be highly enriched in dopaminergic neurons in the substantia nigra [2]. Furthermore, a

point mutation in the gene for the dopamine D2 receptor was found in a family with M-D [23]. We demonstrated ε-SG expression in the dopaminergic neurons of the substantia nigra in mouse brain (Fig. 7). Biochemical fractionation of the brain homogenates suggested that some population of ε-SGs was present in synaptic membranes. These results raise the possibility that ε-SGs might be involved in the neuronal functions through dopaminergic transmission. However, besides the dopaminergic neurons, we detected ε-SG expression in neuronal cells in various brain regions (Figs. 6 and 7). Further studies for the expression of ε-SG among other types of neurons are necessary to elucidate the precise role of ε-SG in the central nervous system. A more recent study reported an M-D family with a novel mutation in the *SGCE* gene associated with epilepsy and/or electroencephalogram (EEG) abnormalities [15]. Interestingly, we found marked expression of ε-SGs in the cerebral cortex, hippocampus, and cerebellar cortex (Figs. 6 and 7), the location of lesions for some types of epilepsy [6,10,28]. It is therefore of interest to study the ε-SG expression in experimental models of epilepsy.

Disruption of the SGC including ε-SG1 does not produce obvious abnormalities in the peripheral nervous system [20], while it does induce vascular smooth muscle irregularities, which eventually cause cardiomyopathy [7]. Biochemical analysis for SGC formation [20] suggests that the loss-of-function mutations in the *SGCE* gene, reported in almost all M-D patients, would disrupt SGCs in the smooth muscle and Schwann cells. However, heart failure has not been reported in M-D patients, suggesting that differences exist between the pathophysiological mechanisms in the brain and smooth muscle in sarcoglycan deficiency.

In summary, we cloned and subsequently characterized two ε-SG isoforms, designated ε-SG1 and ε-SG2, in the brain. Our results strongly suggest that these isoforms play key functional roles in synaptic membranes of neuronal cells. Further investigation into the individual role of ε-SG isoforms would contribute to the understanding of the molecular mechanisms of M-D.

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α1-Syntrophin Modulates Turnover of ABCA1*

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ABCA1 (ATP-binding cassette transporter A1) mediates the release of cellular cholesterol and phospholipid to form high density lipoprotein. Functions of ABCA1 are highly regulated at the transcriptional and post-transcriptional levels, and the synthesized ABCA1 protein turns over rapidly with a half-life of 1-2 h. To examine whether the functions of ABCA1 are modulated by associated proteins, a yeast two-hybrid library was screened with the C-terminal 120 amino acids of ABCA1. Two PDZ (PSD95-Discs large-ZO1) proteins, α1-syntrophin and Lin7, were found to interact with ABCA1. Immunoprecipitation revealed that α1-syntrophin interacted with ABCA1 strongly and that the interaction was via the C-terminal three amino acids SYV of ABCA1. Co-expression of α1-syntrophin in human embryonic kidney 293 cells retarded degradation of ABCA1 and made the half-life of ABCA1 five times longer than in the cells not expressing α1-syntrophin. This effect is not common among PDZ-containing proteins interacting with ABCA1, because Lin7, which was also found to interact with the C terminus region of ABCA1, did not have a significant effect on the half-life of ABCA1. Co-expression of α1-syntrophin significantly increased the apoA-I-mediated release of cholesterol. ABCA1 was co-immunoprecipitated with α1-syntrophin from mouse brain. These results suggest that α1-syntrophin is involved in intracellular signaling, which determines the stability of ABCA1 and modulates cellular cholesterol release.

Cholesterol is not catabolized in the peripheral cells and, therefore, is mostly released and transported to the liver for conversion to bile acids to maintain cholesterol homeostasis. The same pathway may also remove cholesterol that has pathologically accumulated in cells, such as at the initial stage of atherosclerosis. The assembly of high density lipoprotein (HDL)[1] particles by lipid-free apolipoproteins with cellular lipid has been recognized as one of the major mechanisms for the cellular cholesterol release (1, 2). ApoA-I-mediated cholesterol efflux is a major event in "reverse cholesterol transport," a process that generates HDL and transports excess cholesterol from the peripheral tissues, including the arterial wall, to the liver for biliary secretion. The importance of ABCA1 in this active cholesterol-releasing pathway for regulating cholesterol homeostasis became apparent with the finding that it is impaired in the cells from patients with Tangier disease, a genetic deficiency of circulating HDL (3, 4). Tangier disease is caused by mutations in ABCA1. ABCA1 mutations are also a cause of familial HDL deficiency and are associated with premature atherosclerosis (5, 6).

Cholesterol is a prerequisite for cells, but, at the same time, the hyper-accumulation of cholesterol is harmful to cells. Therefore, the expression of ABCA1 is highly regulated at both the transcriptional and post-transcriptional level. The transcription of ABCA1 is regulated by the intracellular oxysterol concentration via the LXR/RXR nuclear receptor (7), and the synthesized ABCA1 protein turns over rapidly with a half-life of 1–2 h (8–10). However, the post-translation regulatory mechanism of ABCA1 is unclear. We analyzed the associated proteins that could be involved in the post-translational regulation of ABCA1. By yeast two-hybrid screening with the C-terminal 120 amino acids of ABCA1, two PDZ (PSD95-Discs large-ZO1)-binding proteins, α1-syntrophin and Lin7, were found to interact with ABCA1. Immunoprecipitation confirmed the association of α1-syntrophin and ABCA1 via its C-terminal amino acids. The importance of this interaction in the regulation of ABCA1 function was examined.

EXPERIMENTAL PROCEDURES

Materials—The anti-ABCA1 monoclonal antibody KM3073 was generated against the first extracellular domain of the human ABCA1 protein in rats. Anti-ABCA1 monoclonal antibody KM3110 was generated against the C-terminal 20 amino acids of ABCA1 in mice. Anti-ABCA1 polyclonal antibody, previously described (11), was used for immunostaining. Affinity-purified antibody specific for α1-syntrophin was prepared using recombinant proteins. Human α1-syntrophin (amino acids 169–346) was fused to glutathione *S*-transferase (GST) in the pGEX vector (Amersham Biosciences) and to the maltose-binding protein (MBP) in the pMAL-c2 vector (New England Biolabs, Inc.). The GST-α1-syntrophin protein was used as an antigen. Obtained rabbit antiserum was affinity purified with the column coupled with the MBP-α1-syntrophin fusion protein. Anti-FLAG epitope monoclonal antibody M2 was purchased from Sigma. Human apoA-I was a gift from Dr. Shinji Yokoyama, Nagoya City University Graduate School of Medical Sciences.

Animals—16-week-old α1-syntrophin (−/−) (12) and wild-type C57BL/6 mice were used in this study. The animals were allowed *ad libitum* access to food and drinking water. Mice carrying mutations were identified by Southern blot analysis as described (12).

Yeast Two-hybrid Library Screening—The Matchmaker Two-hybrid

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[1] The abbreviations used are: HDL, high density lipoprotein; ABCA1, ATP-binding cassette transporter A1; HEK293, human embryonic kidney 293; LXR/RXR, liver X receptor/retinoid X receptor; PBS, phosphate-buffered saline; PDZ, PSD95-Discs large-ZO1.

System 3 from Clontech was used following the manufacturer's instructions. The ABCA1 C terminus region coding for 120 amino acids was cloned from cDNA (13) in pGBKT7. The yeast strain AH109, transformed with pGBKT7/ABCA1-C120, was mated with the yeast strain Y187, which had been pretransformed with a human bone marrow cDNA library. The plasmids, purified from β-galactosidase positive clones, were transformed into *Escherichia coli* and sequenced.

Cellular Lipid Release Assay—Cells were subcultured in poly-L-Lys-coated 6-well plates at a density of 1.0×10^6 cells in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum. After 24 h, cells were transfected with ABCA1 and/or FLAG-α1-syntrophin using LipofectAMINE (Invitrogen). After 24 h of incubation, the cells were washed with phosphate-buffered saline (PBS) and incubated in 0.02% bovine serum albumin in Dulbecco's modified Eagle's medium with 10 μg/ml apoA-I. The lipid content in the medium was determined after 24 h incubation as described previously (14).

Immunoprecipitation Analysis—HEK293 cells, transiently expressing ABCA1, were lysed with PBS containing 1% Triton X-100 and protease inhibitors (100 μg/ml 4-(amidino)-phenylmethanesulfonyl fluoride hydrochloride (pAPMSF)), 10 μg/ml leupeptin, and 2 μg/ml aprotinin). Equal amounts of total protein were incubated with 5 μg of anti-FLAG antibody M2 for 1 h at 4 °C. Brain of normal mice and mice lacking α1-syntrophin (α1-*Syn*$^{-/-}$) (12) was homogenized in ice-cold homogenization buffer (10 mM sodium phosphate, 0.4 M NaCl, 5 mM EDTA, pH 7.8, and the protease inhibitors). The particulate fraction was pelleted by centrifugation (12,000 × *g* for 10 min), resuspended in 10 volumes of homogenization buffer, and recentrifuged. Washed pellets were solubilized in homogenization buffer containing 1% Triton X-100 and incubated on ice for 30 min. The suspension was centrifuged again, and the supernatant was then incubated with anti-α1-syntrophin rabbit polyclonal antibody for 100 min at 4 °C. The immunocomplexes were incubated with protein G-Sepharose (Sigma) for 1 h and washed four times with homogenization buffer containing 1% Triton X-100. The bound proteins were separated by SDS-PAGE (7%) and analyzed by immunoblotting using the anti-ABCA1 antibody KM3073 or KM3110.

Immunostaining—HEK293 cells were co-transfected with ABCA1 and FLAG-tagged α1-syntrophin or FLAG-tagged Lin7 using LipofectAMINE. The cells were fixed in 4% paraformaldehyde and 5% sucrose in PBS$^+$ (PBS with 0.87 mM $CaCl_2$ and 0.49 mM $MgCl_2$) for 30 min and permeabilized for 5 min in 0.4% Triton X-100 in PBS$^+$. The cells were blocked with 10% goat serum diluted with PBS$^+$. This was followed by incubation with anti-ABCA1 polyclonal antibody and anti-FLAG M2 antibody. The cells were then stained with Alexa 488-labeled anti-rat IgG antibody and Alexa 564-labeled anti-mouse IgG antibody (Molecular Probes) as secondary antibodies. The fluorescence images were obtained using an Axiovert microscope (Carl Zeiss) equipped with a MicroRadiance confocal laser-scanning microscope (Bio-Rad).

RESULTS

ABCA1 Interacts with Two PDZ-binding Proteins—To search for proteins that are associated with the C-terminal region of ABCA1, a fusion construct of the Gal4 DNA-binding domain with the C-terminal 120 amino acids of human ABCA1 was used as bait for two-hybrid screening. The identified genes contained two PDZ-containing proteins, α1-syntrophin (10 clones) and Lin7 (two clones). To determine whether the interaction between ABCA1 and α1-syntrophin or Lin7 occurs *in vivo*, we transfected FLAG-tagged α1-syntrophin, FLAG-tagged Lin7, or FLAG-tagged vinexin β (15) (as a negative control) together with ABCA1 into HEK293 cells. Lysates prepared from transfected cells were immunoprecipitated with anti-FLAG antibody, and precipitates were evaluated by immunoblotting with anti-ABCA1 antibody. As shown in Fig. 1, ABCA1 was co-immunoprecipitated with FLAG-tagged α1-syntrophin or FLAG-tagged Lin7, but not with FLAG-tagged vinexin β. ABCA1 was not precipitated with mouse IgG control from any of the lysates in which the expression of ABCA1 (Fig. 1) and FLAG-tagged proteins (data not shown) were detected by immunoblotting. More than 25% of the ABCA1 expressed in HEK293 was roughly estimated to be co-immunoprecipitated with FLAG-tagged α1-syntrophin, suggesting strong interaction between ABCA1 and α1-syntrophin (Fig. 2). The interaction between ABCA1 and Lin7 seemed to be weak, because the amount of precipitated ABCA1 with Lin7 was much less than that with α1-syntrophin. The amount of ABCA1 in lysates was consistently higher when co-expressed with α1-syntrophin than with other proteins.

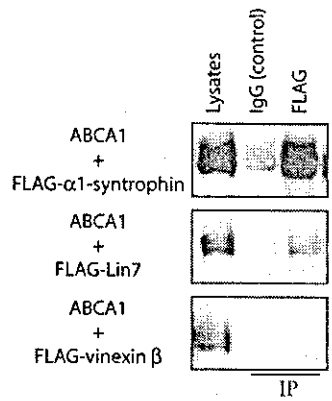

FIG. 1. ***In vivo* association of ABCA1 with α1-syntrophin.** HEK293 cells were co-transfected with human ABCA1 and FLAG-tagged α1-syntrophin, FLAG-tagged Lin7, or FLAG-tagged vinexin β. Cell lysates were immunoprecipitated (*IP*) with anti-FLAG antibody. Immunocomplexes and cell lysates (5%) were subjected to immunoblotting using Anti-ABCA1 monoclonal antibody KM3110, generated against the C-terminal 20 amino acids of ABCA1. Mouse IgG was used as a negative control. The data are representative of three independent experiments.

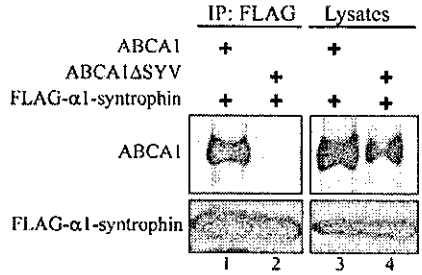

FIG. 2. **ABCA1 interacts with α1-syntrophin via the C-terminal three amino acids.** HEK293 cells were co-transfected with ABCA1 or ABCA1ΔSYV, in which the C-terminal three amino acids were trimmed, and with FLAG-tagged α1-syntrophin. Cell lysates were immunoprecipitated (*IP*) with anti-FLAG antibody. Immunocomplexes and cell lysates (5%) were subjected to immunoblotting using the anti-ABCA1 monoclonal antibody KM3073, generated against the first extracellular domain of the human ABCA1, and an anti-FLAG antibody. The data are representative of two independent experiments.

ABCA1 Interacts with α1-Syntrophin via the C-terminal Three Amino Acids—ABCA1 contains the amino acid sequence ESYV at the C terminus, which has been described as a binding target for syntrophin PDZ domains (16). To determine whether the C-terminal three amino acids SYV are important for the interaction, ABCA1ΔSYV, in which these amino acids were trimmed, was co-expressed with FLAG-tagged α1-syntrophin in HEK293 cells. Although the expression of ABCA1ΔSYV was detected in the lysates, no ABCA1ΔSYV was co-precipitated with FLAG-tagged α1-syntrophin. These results suggest that the interaction is mediated with the C-terminal three amino acids SYV of ABCA1 and α1-syntrophin PDZ domains.

Co-localization of the ABCA1 and PDZ-containing Proteins α1-Syntrophin and Lin7—ABCA1 is mainly localized to plasma membrane but is also substantially expressed in intracellular compartments (11, 17–20). To determine whether ABCA1 and α1-syntrophin or Lin7 are co-localized in cells, ABCA1 was co-transfected with FLAG-tagged α1-syntrophin or FLAG-tagged Lin7 into HEK293 cells. The subcellular localization of these proteins was examined under a confocal laser scanning microscope. α1-Syntrophin resided mainly on plasma mem-

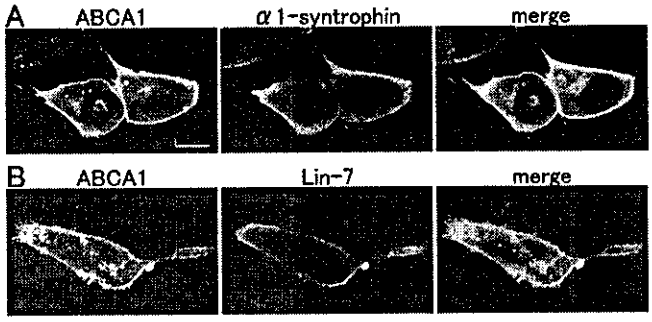

FIG. 3. **Co-localization of ABCA1 and PDZ-containing proteins, α1-syntrophin and Lin7.** HEK293 cells were co-transfected with ABCA1 and FLAG-tagged α1-syntrophin or FLAG-tagged Lin7. The cells were fixed in 4% paraformaldehyde and 5% sucrose, permeabilized in 0.4% Triton X-100, and then doubly stained with anti-ABCA1 polyclonal antibody (*left*) and anti-FLAG antibody (*middle*). A merged image of the staining (*green*, ABCA1; *red*, α1-syntrophin) is also shown (*right*). The data are representative of three independent experiments. *Bar*, 10 μm

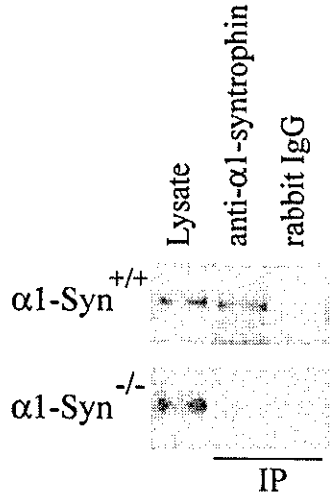

FIG. 4. **Physiological association of mouse ABCA1 with α1-syntrophin.** Brain lysates prepared from normal mouse or α1-syntrophin (−/−) were immunoprecipitated (*IP*) with anti-α1-syntrophin antibody. Immunocomplexes and cell lysates (1%) were subjected to immunoblotting using anti-ABCA1 antibody KM3110. Rabbit IgG was used as a negative control. The data are representative of three independent experiments.

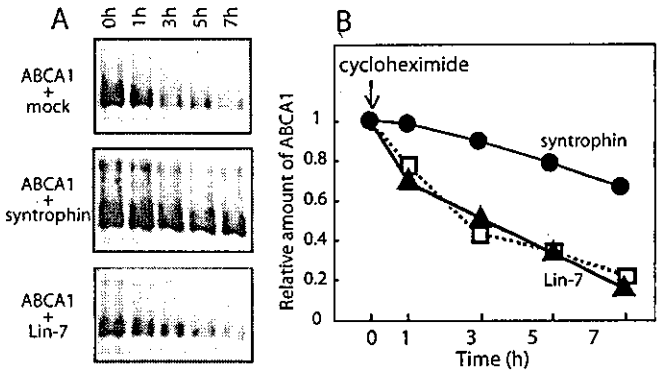

FIG. 5. **α1-Syntrophin modulates turnover of ABCA1.** *A*, HEK293 cells were co-transfected with ABCA1 and vector (*mock*), FLAG-tagged α1-syntrophin, or FLAG-tagged Lin7. At 48 h after transfection, 100 μg/ml of cycloheximide was added to block protein synthesis. After the indicated times, cell lysates were subjected to immunoblotting using Anti-ABCA1 monoclonal antibody KM3110. *B*, quantitation of ABCA1 levels. Values are expressed as fold increase with respect to the amount of ABCA1 just before adding cycloheximide. □, mock transfected; ●, co-transfected with α1-syntrophin; ▲, co-transfected with Lin7. The data are representative of two experiments with similar results.

brane, where it co-localized with ABCA1 (Fig. 3*A*). Lin7 also localized mainly on plasma membrane and appeared not to be uniformly distributed but rather clustered in a specific region of plasma membrane in some cells. In those regions, high expression of ABCA1 and the formation of filopodia were observed (Fig. 3*B*).

Interaction of α1-Syntrophin with ABCA1 in Mouse Brain—Among syntrophin isoforms, α1-syntrophin is mainly expressed in brain, skeletal muscle, and heart in mouse (21). To examine whether ABCA1 and α1-syntrophin interact physiologically, we tried co-immunoprecipitation of these two proteins from mouse brain. Lysates prepared from mouse brain were immunoprecipitated with anti-α1-syntrophin antibody, and precipitates were evaluated by immunoblotting with anti-ABCA1 antibody. As shown in Fig. 4, mouse ABCA1 was co-immunoprecipitated with α1-syntrophin, but not with control IgG. This interaction was confirmed to be specific, because ABCA1 was not precipitated from brain of α1-*Syn*$^{-/-}$ mice (Fig. 4).

α1-Syntrophin Modulates Turnover of ABCA1—Syntrophins have been reported to be involved in protein stability. For example, interaction with β2-syntrophin controls the degradation of ICA512, which connects insulin secretory granules to the utrophin complex and the actin cytoskeleton, by calpain (22), and the stability of AQP4 (23) and neuronal nitric-oxide synthase (12) is suggested as being controlled by α1-syntrophin. The amount of ABCA1 in lysates was consistently higher on co-expression with α1-syntrophin than with other proteins as shown in Fig. 1. Therefore, we examined the effect of α1-syntrophin on the stability of ABCA1. FLAG-tagged α1-syntrophin or FLAG-tagged Lin7 was transiently co-expressed with ABCA1 in HEK293 cells. At 48 h after transfection, the medium was replaced with 10% fetal bovine serum/Dulbecco's modified Eagle's medium containing 100 μg/ml cycloheximide, and cellular protein synthesis was inhibited to block supply of the newly synthesized ABCA1. After the indicated times, the amount of ABCA1 was measured by immunoblotting (Fig. 5*A*). After the inhibition of cellular protein synthesis, 80% of ABCA1 was degraded in 7 h, and the half-life was about 2 h as reported previously (10). Thus, ABCA1 protein turns over rapidly in HEK293 cells. When ABCA1 was co-expressed with α1-syntrophin, only ~30% of ABCA1 was degraded in a 7-h treatment with cycloheximide, and the half-life was estimated to be 10 h (Fig. 5*B*). Lin7, which is a PDZ protein and also binds to the C terminus region of ABCA1, did not show a significant effect on the half-life of ABCA1. The half-life of ABCA1ΔSYV was scarcely affected by co-expression of α1-syntrophin (data not shown). These results suggest that α1-syntrophin decreases ABCA1 protein degradation by interacting with the C terminus three amino acids of ABCA1.

α1-Syntrophin Increases apoA-I-mediated Cholesterol Efflux by ABCA1—To analyze the functional consequences of decreased ABCA1 protein degradation in the presence of α1-syntrophin, the apoA-I-mediated release of cholesterol was examined from HEK293 cells transiently cotransfected with ABCA1 and α1-syntrophin (Fig. 6). Human ABCA1 transiently expressed in HEK293 cells supported the apoA-I-mediated release of cholesterol as previously reported with ABCA1-green fluorescent protein (13). Co-expression of α1-syntrophin significantly increased the apoA-I-mediated release of cholesterol, although expression of α1-syntrophin alone did not affect it.

DISCUSSION

In this study, we identified α1-syntrophin as a protein interacting strongly with ABCA1 via the C-terminal three amino

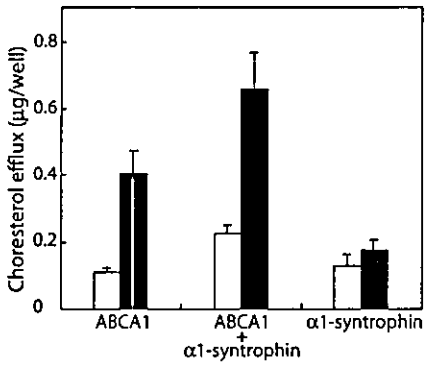

FIG. 6. **Effects of α1-syntrophin co-expression on apoA-I-mediated cholesterol transport.** The cholesterol content of the medium in 6-well plates containing HEK293 cells transiently transfected with ABCA1 alone, ABCA1 and α1-syntrophin, and α1-syntrophin alone was measured after 24 h incubation in the presence (*black bars*) or absence (*white bars*) of 10 μg/ml of apoA-I.

acids SYV of ABCA1. Co-expression of α1-syntrophin retarded degradation of ABCA1 and made the half-life of ABCA1 in HEK293 cells five times longer than in the cells not expressing α1-syntrophin. This effect is not common among PDZ-containing proteins interacting with ABCA1, because Lin7, which also binds to the C terminus region of ABCA1, did not show a similar effect. Co-expression of α1-syntrophin significantly increased the apoA-I-mediated release of cholesterol. Because this interaction was observed in mouse brain, α1-syntrophin could be involved in lipid homeostasis in brain.

Mammalian cells have developed sophisticated mechanisms to ensure adequate cellular cholesterol levels, because cholesterol plays a critical role in several important cell functions, including protein trafficking, membrane vesiculation, and signal transduction, and, at the same time, hyper-accumulation of cholesterol is harmful for cells. Plasma membrane cholesterol content, for example, is regulated through a feedback mechanism controlled by sterol regulatory element binding protein-2 (SREBP-2) (24, 25). To eliminate excess cholesterol from the cell, expression of ABCA1, a key molecule for apoA-I-mediated cholesterol efflux, is stimulated by intracellular oxysterol via the LXR/RXR nuclear receptor (26). The synthesized ABCA1 protein turns over rapidly with a half-life of 1–2 h (8, 10) to cancel cholesterol efflux by ABCA1. Because co-expression of α1-syntrophin retarded degradation of ABCA1 and made the half-life of ABCA1 in HEK293 cells five times longer than in the cells not expressing α1-syntrophin, α1-syntrophin is expected to be involved in intracellular signaling, which determines the stability of ABCA1.

Recently, it has been proposed that ABCA1 is regulated in two different ABCA1 degradation pathways under various cellular conditions: (i) a basal calpain degradation pathway that is turned off by interaction with apolipoproteins (9, 10); and (ii) a ubiquitin-proteasome pathway that is activated by marked free cholesterol loading (27). A sequence rich in proline, glutamate, serine, and threonine (PEST sequence) just before the second membrane spanning domain of ABCA1 (amino acid residue 1283–1306) is involved in regulating the calpain degradation of ABCA1 (10). Although the nature of the apoA-I-ABCA1 interaction is not fully understood, conformational alteration of ABCA1 through the PEST sequence may be induced by its direct or indirect interaction with apoA-I, which may render ABCA1 resistant to proteolysis by calpain. Because ABCA1 was ubiquitinated as well when co-expressed with α1-syntrophin (data not shown), α1-syntrophin seems not to affect ABCA1 degradation in an ubiquitin-proteasome pathway. Binding of α1-syntrophin to the C terminus of ABCA1 may cause a conformational alteration similar to that caused by apoA-I binding and render ABCA1 resistant to proteolysis by calpain.

Syntrophins are a family of five proteins (α1, β1, β2, γ1, and γ2,) containing two pleckstrin homology domains, a PDZ domain, and a C-terminal syntrophin-unique region (28). Analysis of α-*Syn*$^{-/-}$ mouse has demonstrated that perivascular localization of AQP4 in brain requires α1-syntrophin (23) and that the stability of AQP4 (23) and neuronal nitric-oxide synthase (12) decreases in the absence of α1-syntrophin. β2-syntrophin was also reported to interact with ABCA1 and was proposed to participate in the retaining of ABCA1 in cytoplasmic vesicles by forming a ABCA1-β2-syntrophin-utrophin complex (29). It is possible that α1-syntrophin is also involved in endocytotic recycling of ABCA1. Extracellular lipid-free apoA-I may first interact with ABCA1 on plasma membrane, but it is not clear whether the formation of HDL takes place extracellularly or if intracellular events, such as endocytotic recycling, are involved (30). It is intriguing that apoA-I and α1-syntrophin have a similar effect on ABCA1 turnover. A mutation of ABCA1 that causes Tangier disease (W590S) does not affect apoA-I binding or initial ATP binding/hydrolysis but results in a defect in lipid efflux (11, 31, 32). Because apoA-I failed to affect calpain degradation of ABCA1-W590S in HEK293 cells (10), additional signals following apoA-I binding to ABCA1 are speculated to be necessary for the subsequent inhibition of calpain degradation. It is possible that PDZ-containing proteins such as α1-syntrophin are involved in intracellular signaling, which determines the stability of ABCA1.

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Up-regulation of IL-4 production by the activated cAMP/cAMP-dependent protein kinase (protein kinase A) pathway in CD3/CD28-stimulated naive T cells

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Abstract

The signal transduction of the cAMP/cAMP-dependent protein kinase [protein kinase A (PKA)] pathway through multiple receptors is critical for many processes in all cell types. In T cells, the engagement of both the TCR–CD3 complex and the CD28 co-stimulatory molecule also induces cAMP, and subsequently activates PKA. It is believed that elevation of cAMP levels in T cells is inhibitory of IL-2 production and T cell proliferation. However, the function and detailed signal transduction mechanisms of the cAMP/PKA pathway in naive T_h cells are less well understood. In this study, we show that calcitonin gene-related peptide (CGRP) down-regulates IL-2 and IFN-γ production and up-regulates IL-4 production to promote T_h2 differentiation by moderate activation of the cAMP/PKA pathway via the CGRP receptor in the presence of a CD3/CD28 co-stimulation signal. The IL-4 production and transcriptional activation of T_h2 cytokine mRNAs were also reproduced by the addition of a cAMP analogue, dibutyryl-cAMP, in CD3/CD28-stimulated naive T_h cells. More interestingly, cAMP/PKA activation in naive T_h cells stimulated with anti-CD3 plus anti-CD28 mAb is essential for inducing IL-4 production and promoting T_h2 differentiation; in addition, NF-AT is a downstream effector of the cAMP/PKA signaling pathway. These findings indicate that the cAMP/PKA pathway transduces the critical activation signal to T_h2 polarization by a CD3/CD28 co-stimulation signal and a PKA activating reagent.

Introduction

Optimal activation of T cells requires both the engagement of the TCR–CD3 complex, and interactions between co-stimulatory molecules such as CD28 (1) and ligands B7-1 (CD80) and B7-2 (CD86), which are expressed on antigen-presenting cells (APC) (2–4). Stimulation of the TCR–CD3 complex in the absence of the CD28-mediated co-stimulatory signal drives T cells to enter a state of anergy or else to undergo apoptosis (5–7). Conversely, induction of T cell anergy can be prevented by stimulation of CD28 with B7 molecules or anti-CD28 antibody (8). Moreover, the CD28-mediated co-stimulatory signal in T cells augments the proliferation and secretion of multiple T cell cytokines such as IL-2, IFN-γ and IL-4 (9,10).

Murine $CD4^+$ T cells have been divided into functionally distinct subsets based on their cytokine production profiles. T_h1 cells produce IL-2 and IFN-γ, whereas T_h2 cells produce IL-4, IL-5, IL-10 and IL-13. T_h cell-derived cytokines participate in the development and progression of a number of disease states, direct normal immune responses, and regulate naive $CD4^+$ T cell differentiation. It is, therefore, essential to understand the signal transduction mechanisms that regulate cytokine production in T_h cells.

In recent years, understanding of TCR–CD3 signal transduction has progressed significantly. On the other hand, CD28-mediated co-stimulatory signals are not well

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understood; however, some key molecules that transduce CD28 signaling have been identified. CD28 co-stimulation signal induces phosphorylation and activation of a proto-oncogene Vav (11). Vav acts as a guanine-nucleotide exchange factor for the small Rho family GTPases Rac and CDC42, allowing these molecules to switch from the inactive GDP-bound state to an active, GTP-bound state. Active forms of Rac and CDC42 then activate p21-activated kinase, leading to the activation of extracellular signal-regulated protein kinase (ERK) pathway. Other mitogen-activated protein kinase (MAPK) pathways, i.e. c-Jun N-terminal kinase (JNK) and p38 MAPK, appear to be involved in signal transduction of the CD28 co-stimulatory molecule as well. ERK and JNK phosphorylate c-*fos* and c-*jun* proteins respectively, which assemble to form the activated protein-1 (AP-1) transcription factor. AP-1 binds to the IL-2 promoter and then induces IL-2 production for T cell proliferation (12,13). Additionally, JNK has been shown to play an important role in IL-2 mRNA stabilization (14). Moreover, p38 MAPK has been reported to be preferentially required for T_h2-type responses in human $CD4^+CD45RO^+$ T cells and T_h2 effector cells (15).

There is growing evidence that the nervous system and the immune system cross-talk through common molecules and their receptors. In a previous study, we demonstrated that calcitonin gene-related peptide (CGRP), a 37-amino-acid neuropeptide, modulates the profile of IFN-γ and IL-4 production from mouse splenocytes and T_h cell clones stimulated with the appropriate antigen (16). CGRP plays important roles as a neurotransmitter/neuromodulator in the central nervous system and as a potent vasodilator when secreted from peripheral, perivascular nerves (17,18). These physiological functions are mediated through its binding to a specific receptor composed of calcitonin receptor-like receptor (CRLR) and receptor activity modifying protein 1 (RAMP1) (19). CGRP and its receptor are found to be expressed in various organs such as brain, lung, thyroid, thymus and spleen (20,21). Recently, we cloned the mouse cDNA counterpart of the human CGRP receptor composed of CRLR and RAMP1, and clarified expression of the CGRP receptor on T cells (22). These results indicate that signaling through CGRP receptors modulates signal transduction by engagement of TCR and CD28 in T cells. Since CGRP receptors are coupled with $G_s\alpha$ protein (23), we hypothesized that the cAMP/PKA pathway is likely to be involved in the regulation of cytokine production by CGRP in activated T cells.

We report here that CGRP inhibited IL-2 and IFN-γ production, and augmented IL-4 production to promote T_h2 differentiation by activation of the cAMP/PKA pathway in the presence of a CD3/CD28 co-stimulation signal. The IL-4 production and transcriptional activation of T_h2 cytokine mRNAs were also reproduced by dibutyryl-cAMP (db-cAMP) in CD3/CD28-stimulated naive T_h cells. Moreover, the CD3/CD28 co-stimulation signal also activates the cAMP/PKA pathway and then enhances NF-AT transcription activity to skew immune responses to the T_h2 phenotype.

Methods

Mice

Specific pathogen-free male 5- to 7-week-old BALB/c mice were purchased from Charles River Japan (Kanagawa, Japan). Ovalbumin (OVA)-specific TCRαβ transgenic mice (DO 11.10 Tg) were kindly distributed by Dr Hiromichi Ishikawa (Keio University, Tokyo, Japan).

Antibodies and reagents

Anti-mouse IL-2 mAb (MM-101 and MM102-B) and anti-mouse IFN-γ mAb (MM701) were purchased from Pierce (Rockland, IL). Biotinylated anti-mouse mouse IL-4 (BVD6-24G2), biotinylated anti-mouse mouse IFN-γ (XMG1.2), anti-IFN-γ (XMG1.2)–FITC and anti-IL-4 (11B11)–phycoerythrin were purchased from BD PharMingen (San Diego, CA). The mAb to CD8 (3–155), B220 (RA3.3), MHC class II (34–5.3), Thy-1.2 (HO13.4.9) and CD3 (2C11, OKT3) were obtained from ATCC (Rockville, MA). The antigenic OVA synthetic peptide (residues 323–339; ISQAVHAAHAEINEAGR) was synthesized by Fuso Pharmaceutical Industries (Osaka, Japan). CGRP was purchased from the Peptide Institute (Osaka, Japan), db-cAMP was purchased from Wako (Osaka, Japan), *N*-[2-((*p*-bromocinnamyl)aminoethyl)]-5-isoquinolinesulfonamide (H-89) was purchased from Sigma (St Louis, MO). PKI_{14-22} was purchased from Calbiochem (La Jolla, CA). The pNF-AT72-luc was kindly provided by Dr Kenichi Arai (University of Tokyo, Tokyo, Japan); $p(Ig\kappa)_3$-luc was the generous gift of Dr Tatsuhiko Furukawa (Kagoshima University, Kagoshima, Japan). $pCMV_T$-TK neo PKA-R^{G324D} was kindly provided by Dr Tania H. Watts (University of Toronto, Toronto, Ontario, Canada) (24). PathDetect *in vivo* signal transduction pathway reporting systems [c-Jun *trans*-, Elk *trans*-, CHOP *trans*- and cAMP response element binding protein (CREB) *trans*-reporting systems] were purchased from Stratagene (La Jolla, CA). pSV-β-galactosidase control vector was obtained from Promega (Madison, WI).

Preparation and stimulation of naive $CD4^+$ T cells

To isolate naive $CD4^+$ T cells, mouse splenocytes were incubated with anti-CD8 mAb (3–155), anti-B220 mAb (RA3.3) and anti-MHC class II (34–5.3) mAb at 4°C for 30 min, and then were incubated on anti-mouse Ig-coated plates to eliminate B cells and $CD8^+$ T cells. The $CD4^+$ T cell-enriched population contained >80% $CD4^+$ T cells. APC were prepared from splenic cells by cytotoxic killing treatment with anti-Thy-1.2 mAb (HO13.4.9) and a rabbit complement (Rockland, Gilbertsville, PA). The $CD4^+$ T cells (10^6 cells/ml) were stimulated with antigenic peptide ($OVA_{323-339}$) in the presence of the splenic APC (5×10^6 cells/ml) or with plate-coated anti-CD3 mAb (2C11) and/or soluble anti-CD28 mAb (PV-1).

Measurement of cytokine concentrations

Purified $CD4^+$ T cells were cultured on mAb-coated plates for 48 h and the cytokine concentrations in culture supernatants determined by ELISA. An in-house ELISA was employed to quantify IL-2, IFN-γ and IL-4 as follows. Microtiter wells were coated overnight with anti-mouse IL-2, anti-mouse IFN-γ and anti-mouse IL-4 mAb. Samples and the standards were

diluted in RPMI 1640 supplemented with 2% FCS and incubated for 2 h at 37°C. After washing with PBS/0.05% Tween 20, each well was incubated for 1 h with biotinylated anti-mouse IL-2, anti-mouse IFN-γ and anti-mouse IL-4 mAb, washed, incubated for 30 min with horseradish peroxidase-conjugated streptavidin (Amersham Pharmacia Biotech, Little Chalfont, UK), and developed with 2,2′-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid) (Kirkegaard & Perry, Gaithersburg, MD). Absorbance was determined at a wavelength of 405 nm. Quantities of IL-2, IFN-γ and IL-4 were calculated according to the standard curves. The lower limit of detection was 10 U/ml for IL-2, 150 pg/ml for IFN-γ and 6 pg/ml for IL-4.

Proliferation assays

CD4+ T cells (10^5 cells/well) in a 96-well plate were stimulated with plate-coated anti-CD3 mAb or plate-coated anti-CD3 plus soluble anti-CD28 mAb in the presence or absence of 1 nM CGRP for 18 h. Cells were pulsed with 1 μCi [^{3}H]thymidine (Amersham Pharmacia Biotech) for 6 h, harvested and counted with a scintillation counter (Packard, Meriden, CT).

Measurement of cAMP amount

CD4+ T cells (10^6 cells/well) in a 96-well plate were stimulated with anti-CD3 mAb, anti-CD28 mAb or CGRP. After 15 min incubation, the cells were lysed with 20 μl lysis buffer. cAMP levels in the lysate were measured by the non-acetylation method using an enzyme immunoassay system (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Cytokine mRNA analysis

The relative mRNA expression of 23 cytokines was analyzed with GEArray (SuperArray, Bethesda, MD) according to the manufacturer's protocol. In brief, CD4+ T cells (10^6 cells/ml) were stimulated with 1 μg/ml anti-CD3 plus 1 μg/ml anti-CD28 mAb in the presence or absence of 5 μM db-cAMP. After 48 h incubation, total RNA was extracted with TRIzol reagent (Gibco/BRL, Grand Island, NY). Total RNA (5 μg) from each sample was reverse transcribed into cDNA with MMLV reverse transcriptase (Gibco/BRL) in the presence of [α-^{32}P]dCTP (Amersham Pharmacia Biotech). The synthesized cDNA probes were hybridized to cytokine gene-specific cDNA fragments that were spotted on the GEArray membrane. The amount of radioactive signaling from the hybridized probes was analyzed with a BAS1500 phosphoimager (Fuji, Kanagawa, Japan). The signal from expression of each cytokine gene was normalized to the signal derived from GAPDH on the same membrane and expressed as cytokine mRNA arbitrary units. These units were calculated with the following formula. Cytokine mRNA arbitrary units = (cytokine signal − background signal)/(GAPDH signal − background signal) × 100.

In vitro PKA assay

CD4+ T cells (10^6 cells/well) in a 48-well plate were stimulated with 1 nM CGRP and/or 10 μg/ml plate-coated anti-CD3 plus 1 μg/ml soluble anti-CD28 mAb. After 1 h incubation, the cells were lysed in the lysis buffer containing 1 μM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1.8 μg/ml iodoacetamide, and PKA activity in lysates was determined by a PKA radioimmunoassay kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer's instructions.

Induction of T_h cells and intracellular cytokine staining

CD4+ T cells (10^6 cells/well) in a 48-well plate were stimulated with plate-coated anti-CD3 mAb and soluble anti-CD28 mAb in the presence or absence of CGRP or H-89 for 2 days. After 5 days, the T cells were re-stimulated with anti-CD3 mAb for 4 h in the presence of 2 μM monensin (Sigma). Then, cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X. After blocking with PBS containing 3% BSA, cells were stained with anti-IFN-γ-FITC and anti-IL-4–phycoerythrin, as described previously (25). Flow cytometric analysis was performed on FACSCalibur with CellQuest software (Becton Dickinson).

DNA transfection and luciferase assay

Jurkat cells expressing the mouse CD28 (Jurkat-CD28) were cultured in RPMI 1640 supplemented with 10% FCS (26). Jurkat-CD28 cells were transfected with 5 μg of pNF-AT72-luc or p(Igκ)3-luc for measuring NF-AT or NF-κB activity respectively, or 5 μg of pFR-luc concomitant with 0.5 μg of pFA-CREB, pFA-CHOP, pFA-c-jun or pFA-Elk for measuring CREB, p38, JNK or ERK activities respectively, using a Genepulser (Bio-Rad, Hercules, CA). After a 24-h incubation, the transfected cells were stimulated with plate-coated anti-human CD3 mAb (OKT3) or anti-CD3 plus soluble anti-CD28 (PV-1) mAb. In Fig. 6(B), Jurkat-CD28 cells were co-transfected with 5 μg of pNF-AT72-luc and 2 μg of pSV-β-galactosidase control vector in combination of 15 μg of pCMV$_T$-TK neo vector or pCMV$_T$-TK neo PKA-R^{G324D} vector. A pSV-β-galactosidase control vector was used for normalizing the transfection efficiency. The transfectants were stimulated with anti-CD3 mAb or anti-CD3 plus anti-CD28 mAb for 24 h. The cells were harvested and lysed in cell culture lysis reagent (Promega), followed by measurements of luciferase activity on a Lumat LB9501 luminometer (Berthold, Bundoora, Australia) or β-galactosidase activity.

Gel mobility shift assay

Nuclear extracts were prepared from CD4+ T cells stimulated with 10 μg/ml plate-coated anti-CD3 mAb and 1 μg/ml soluble anti-CD28 mAb in the presence or absence of 1 nM CGRP or 20 μM H-89 for 6 h. Gel mobility shift assays were carried out as outlined by the manufacturer (EMSA kit, NF-ATc probe; Panomics, Redwood City, CA).

Statistical analysis

Results are presented as means ± SD. For the two-group comparison, the statistical significance of differences was determined by Student's *t*-test for unpaired data.

Results

CGRP up-regulates IL-4 production from CD3/CD28-stimulated CD4+ T cells

CGRP is thought to be one of the neuropeptides involved in cross-talk between the neuronal system and the immune

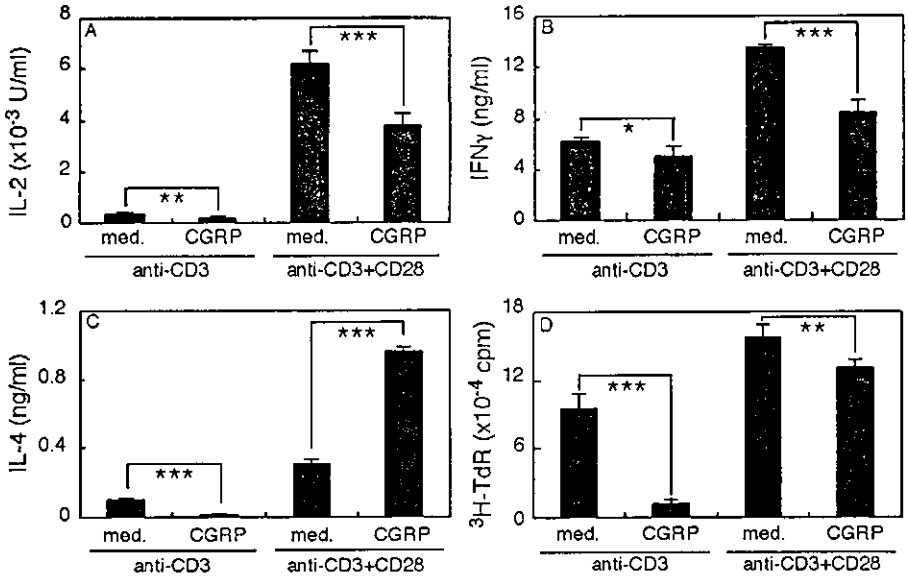

Fig. 1. Effect of CGRP on cytokine production and proliferation of naive CD4+ T cells stimulated with anti-CD3 mAb alone or with anti-CD3 plus anti-CD28 mAb. Naive CD4+ T cells (10^6 cells/ml) were stimulated with 10 μg/ml anti-CD3 mAb alone or 10 μg/ml anti-CD3 plus 1 μg/ml anti-CD28 mAb in the presence or absence of 1 nM CGRP. After a 48-h incubation, concentrations of IL-2 (A), IFN-γ (B) and IL-4 (C) in the culture supernatants were determined by ELISA. For proliferation activity, T cells (2×10^5 cells/well) were plated in 96-well plates and stimulated with anti-CD3 mAb alone or with anti-CD3 plus CD28 mAb in the presence or absence of 1 nM CGRP for 24 h. Six hours after 1 μCi [^{3}H]thymidine addition, T cells were harvested for scintillation counting (D). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

system via its specific receptor. To examine the effect of CGRP on cytokine production and proliferation, we stimulated naive CD4+ T cells with anti-CD3 mAb or anti-CD3 plus anti-CD28 mAb in the presence or absence of 1 nM CGRP. The proliferative responses, and IL-2, IL-4 and IFN-γ secretion were analyzed at 24 and 48 h respectively. When T cells were stimulated with plate-coated anti-CD3 mAb alone, CGRP significantly inhibited the production of IL-2 (20%), IFN-γ (15%) and IL-4 (90%), and T cell proliferation (90%) (Fig. 1). The addition of soluble anti-CD28 mAb clearly augmented the production of three cytokines and T cell proliferation. Under these circumstances, addition of 1 nM CGRP in the presence of CD28 co-stimulatory signal reduced the production of IL-2 and IFN-γ as well as T cell proliferation; however, IL-4 production was significantly increased (3.2-fold) by CGRP (Fig. 1C). CGRP at concentrations of 1–100 nM revealed a dose-dependent effect on cytokine production from CD3/CD28-stimulated T cells (data not shown).

Interaction of B7 and CD28 is essential for up-regulation of IL-4 production by CGRP

Next, we examined the effect of CGRP on initial IL-4 production in antigen-induced responses. For this purpose, CD4+ T cells purified from splenocytes of OVA-specific TCR transgenic mice, DO11.10 Tg, were stimulated with 1 μM $OVA_{323-339}$ peptide and APC in the presence or absence of 1 nM CGRP. As shown in Fig. 2, CGRP augmented IL-4 production as much as 2.5-fold compared to the control T cells, which is a similar result to that achieved by stimulation with anti-CD3 plus anti-CD28 mAb (Fig. 1C).

The B7 family molecules, such as B7-1 and B7-2, expressed on APC bind to CD28 expressed on T cells and this process is critical to induce full activation of T cells in primary responses. To directly examine the relevance of the CD28-derived co-stimulatory signal in the up-regulation of IL-4 production by CGRP, we blocked the B7–CD28 interactions using CTLA-4–Ig, a soluble fusion protein made from the extracellular portion of CTLA-4 linked to the Fc portion of IgG (27). The addition of CTLA-4–Ig completely inhibited up-regulation of IL-4 production by CGRP, indicating that signal transductions through both the CGRP receptor and the CD28 co-stimulatory molecule were coupled to IL-4 production.

Effect of db-cAMP on T_h2 cytokine production from CD3/CD28-stimulated CD4+ T cells

CGRP receptors have been reported to couple with $G\alpha_s$ and $G\beta\gamma$ subunits of G proteins. After binding of CGRP to its receptors, the $G\alpha_s$ subunit activates adenylyl cyclase, thereby increasing intracellular cAMP levels. Therefore, we determined cAMP levels in naive CD4+ T cells stimulated with anti-CD3 mAb and/or anti-CD28 mAb in the presence or absence of CGRP for 15 min (Fig. 3A). CGRP (1 nM) alone induced ~2.5-fold cAMP production compared to that of the control level, but stimulation with anti-CD3 mAb or anti-CD28 mAb did not have this inductive effect. The combination of anti-CD3 plus anti-CD28 mAb markedly increased the cAMP level and this increase was further augmented by the addition of CGRP. The changes in cAMP levels by the stimulation of anti-CD3/CD28 mAb or anti-CD3/CD28 mAb plus CGRP were similar to the changes in IL-4 production (Fig. 1C), although the stimulation with either anti-CD3 mAb or anti-CD28 mAb failed to induce production of both the cAMP elevation and the initial IL-4 production. These results indicated that elevation of the

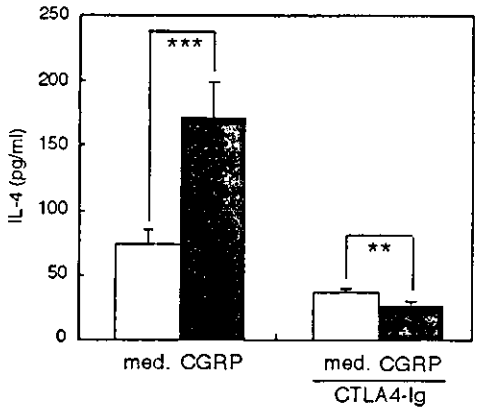

Fig. 2. The CD28 co-stimulation signal is essential for CGRP-induced IL-4 production from antigenic peptide-stimulated T cells. $CD4^+$ T cells (10^6 cells/ml) from DO.11.10 Tg mice were stimulated with 1 μM $OVA_{323-339}$ peptide and APC (5×10^6 cells/ml) in the presence or absence of 1 nM CGRP. CTLA-4-Ig (10 μg/ml) was added to the culture at the same time as the CGRP stimulation was performed. After a 48-h incubation period, IL-4 levels in the culture supernatants were measured by ELISA. Values are means ± SD ($n = 4$). med.: medium. $^{**}P < 0.01$, $^{***}P < 0.001$.

cAMP level plays an important role on the initial IL-4 production induced by CD3/CD28 co-stimulation.

To investigate whether a cAMP analogue, db-cAMP, had the same effect as CGRP on IL-4 production, we stimulated naive $CD4^+$ T cells with anti-CD3 mAb and anti-CD28 mAb in the presence of various concentrations of db-cAMP, and IL-4 production was assayed after 48 h. The addition of db-cAMP reduced IL-4 production from the T cells stimulated with anti-CD3 mAb alone; however, the same treatment increased IL-4 production from T cells stimulated with anti-CD3 plus anti-CD28 mAb in a dose-dependent manner (Fig. 3B). The addition of 5 μM db-cAMP with CD3 stimulation revealed ~45% inhibition of the IL-4 level, whereas the same treatment, together with CD3/CD28 co-stimulation, produced ~2.3-fold increase. On the other hand, db-cAMP inhibited IL-2 and IFN-γ production both with CD3 stimulation alone and in conjunction with CD28 co-stimulation in a dose-dependent manner, although db-cAMP had no effect on the survival of T cells at concentrations <5 μM (data not shown).

Next, we examined the specificity of db-cAMP-dependent cytokines production in naive $CD4^+$ T cells stimulated with anti-CD3/CD28 mAb using a cytokine array. Total RNA was isolated from $CD4^+$ T cells that had been cultured for 48 h with anti-CD3 plus anti-CD28 mAb in the presence or absence of 5 μM db-cAMP. The RNA from each sample was reverse transcribed into cDNA in the presence of [^{32}P]dCTP and the synthesized ^{32}P-labeled cDNAs were hybridized to a GEArray cytokine membrane in which 23 cytokine gene-specific cDNA fragments, including IL-1 to IL-18, granulocyte colony stimulating factor, LT-β, IFN-γ, tumor necrosis factor (TNF)-α and TNF-β, were spotted. The signals from each spot on the membrane were then scanned with a phosphoimager. To eliminate potential variation in RNA quantification between samples, the signal from expression of each cytokine gene was corrected by that of GAPDH (as 100 arbitrary units) on the same membrane and was expressed as an arbitrary unit.

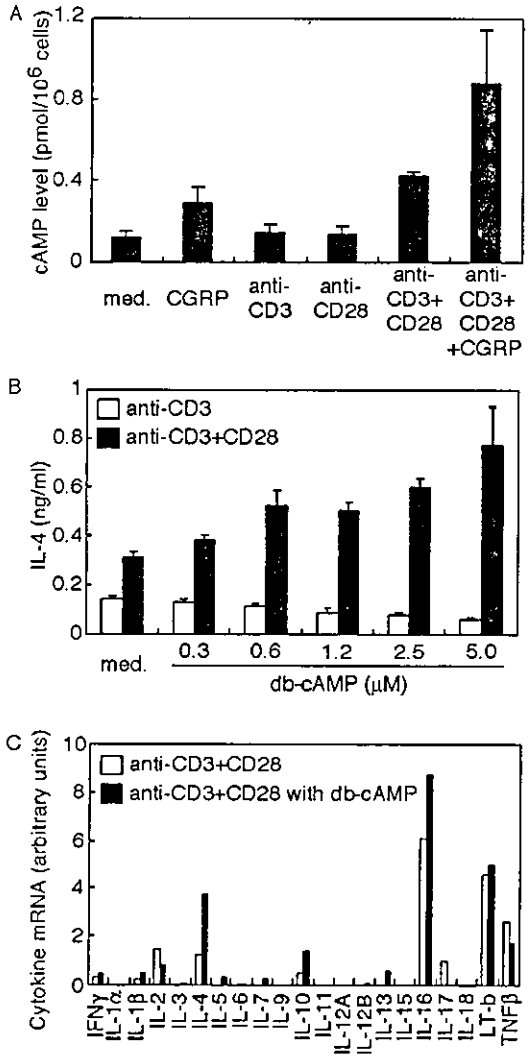

Fig. 3. Effects of db-cAMP on cytokine production of naive $CD4^+$ T cells stimulated with anti-CD3 plus anti-CD28 mAb. (A) Increase in intracellular cAMP levels by CGRP stimulation and/or CD3/CD28 co-stimulation. $CD4^+$ T cells (10^6 cells/ml) were stimulated with the combination of 1 nM CGRP, 10 μg/ml anti-CD3 mAb and 1 μg/ml anti-CD28 mAb. T cells were lysed at 15 min after stimulation and cAMP levels in the lysates were measured by EIA. Values are means ± SD ($n = 3$–6). (B) $CD4^+$ T cells (10^6 cells/ml) were stimulated with 10 μg/ml anti-CD3 mAb alone or 10 μg/ml anti-CD3 plus 1 μg/ml anti-CD28 mAb in the presence of various concentrations of db-cAMP. IL-4 levels in culture supernatants were determined by ELISA. Values are means ± SD ($n = 4$). (C) $CD4^+$ T cells (10^6 cells/ml) were stimulated with 10 μg/ml anti-CD3 plus 1 μg/ml anti-CD28 mAb in the presence or absence of 5 μM db-cAMP. After a 48-h incubation period, total RNA was isolated and reverse transcribed to cDNA in the presence of [α-^{32}P]dCTP. Labeled cDNAs were hybridized to GEArray membranes. Cytokine mRNA levels were calculated as arbitrary units, relevant to GAPDH.

Since GEArray is a semiquantitative method of mRNA expression used for screening purposes, we considered a 3-fold deviation of the signal from the control signal as a significant difference. As shown in Fig. 3(C), the concomitant stimulation of db-cAMP with anti-CD3/CD28 mAb induced marked increases in IL-4, IL-5, IL-10 and IL-13 mRNA from $CD4^+$ T cells; however, the changes in IFN-γ or IL-2 mRNA

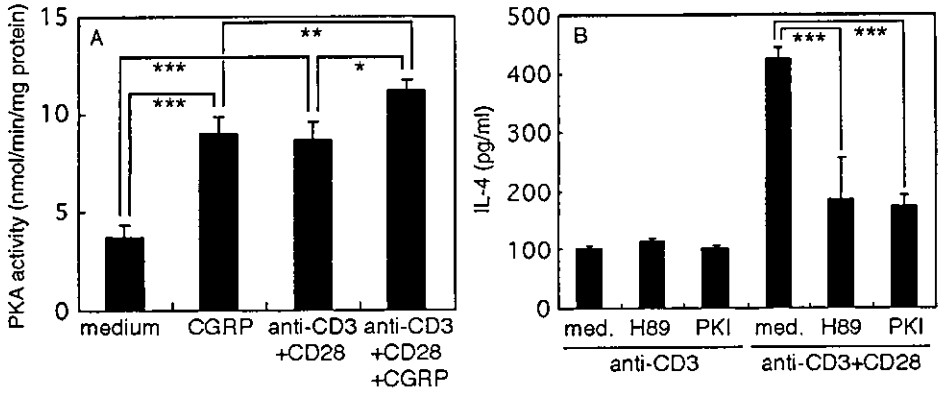

Fig. 4. Effects of PKA on IL-4 production of naive CD4+ T cells stimulated with anti-CD3 plus anti-CD28 mAb. (A) Increase in PKA activity by CGRP stimulation and/or CD3/CD28 co-stimulation. CD4+ T cells were stimulated with 1 nM CGRP and/or 10 μg/ml anti-CD3 plus 1 μg/ml anti-CD28 mAb. T cells were lysed at 1 h after stimulation and the PKA activity in the lysates was determined by radioimmunoassay. Values are means ± SD (n = 4). (B) Inhibitory effects of H-89 and PKI_{14-22} on IL-4 production from CD3/CD28 activated-CD4+ T cells. CD4+ T cells (10^6 cells/ml) were stimulated with 10 μg/ml anti-CD3 mAb alone or 10 μg/ml anti-CD3 plus 1 μg/ml anti-CD28 mAb in the presence or absence of 20 μM H-89 or 30 μM PKI_{14-22}. After 48 h incubation, IL-4 levels in the culture supernatants were determined by ELISA. Values are means ± SD (n = 4). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

levels were neither obvious nor suppressed respectively. We confirmed the transcriptional activity of IL-4, IL-5, IL-10 and IL-13 with semiquantitative RT-PCR in additional experiment (data not shown). Those results were clearly consistent with the results from the cytokine array. It is of note that the cytokine mRNAs increased by db-cAMP stimulation belonged to T_h2 cytokines, indicating that cAMP-mediated signaling is involved in T_h2, but not T_h1, cytokine production.

CD28 co-stimulation signal activates PKA to produce IL-4

cAMP activates PKA to transduce PKA signaling downstream. Since it was observed that CGRP and CD3/CD28 co-stimulation increased cAMP production, we determined the PKA activity by the following method. Naive CD4+ T cells were stimulated with 1 nM CGRP and/or anti-CD3 plus anti-CD28 mAb for 1 h, and PKA activity in cell lysates was measured by a PKA-specific substrate, kemptide. As shown in Fig. 4(A), PKA activity was equally increased by stimulation with CGRP or anti-CD3 plus anti-CD28 mAb. Concomitant stimulation with CGRP and anti-CD3/CD28 mAb further enhanced PKA activity. Since CD3/CD28 co-stimulation induced the elevation of both cAMP levels and PKA activity, we hypothesized that the activation of the cAMP/PKA pathway by CD3/CD28 co-stimulation may play a role to regulate T_h2 cytokine production in CD4+ T cells. To confirm this hypothesis, we first examined the effects of PKA inhibitors, H-89 and PKI_{14-22}, on IL-4 production from CD4+ T cells stimulated with anti-CD3 mAb or anti-CD3 plus anti-CD28 mAb for 48 h. The PKA inhibitors markedly inhibited IL-4 production from T cells stimulated with anti-CD3 plus anti-CD28 mAb, while they had no effect on IL-4 production from T cells stimulated with anti-CD3 mAb alone (Fig. 4B). These results led to the possibility that the cAMP/PKA pathway plays an important role in IL-4 production from CD4+ T cells via CD3/CD28 co-stimulation, but not via CD3 stimulation. On the other hand, the PKA inhibitors did not completely suppress IL-4 production by CD3/CD28 co-stimulation, suggesting that the cAMP/PKA pathway is a major signal transduction pathway for IL-4 production in naive CD4+ T cells; other pathways, such as the Ca^{2+}/calcineurin pathway, are also likely to be involved in IL-4 production.

Involvement of PKA on T_h2 differentiation by CD3/CD28 co-stimulation

CD28 ligation on naive CD4+ T cells was shown to promote T_h2 differentiation *in vivo* (28) as well as *in vitro* (29). Therefore, we studied the effect of H-89 on T_h2 polarization by CD3/CD28 co-stimulation. CD4+ T cells were stimulated with anti-CD3 plus anti-CD28 mAb in the presence or absence of H-89. Following a 7-day culture, the cells were re-stimulated with anti-CD3 mAb, and subjected to intracellular staining for IFN-γ and IL-4. As shown in Fig. 5, CD3 stimulation alone polarized the T_h0 cells toward the T_h1 subset, whereas T cells stimulated with both anti-CD3 plus anti-CD28 mAb were polarized toward the T_h2 subset. The addition of H-89 to T_h0 cells stimulated with anti-CD3 plus anti-CD28 mAb almost completely reversed the effect of the CD28 co-stimulation signal, i.e. IFN-γ production was augmented and IL-4 production was suppressed, resulting in marked block of T_h2 polarization. On the other hand, concomitant addition of 1 nM CGRP with both anti-CD3 and anti-CD28 mAb to T_h0 cells further increased IL-4 production compared to that of the absence of CGRP. These results suggested that the cAMP/PKA pathway mediates IL-4 production in initial T cell activation and is an essential process to generate T_h2 responses via the CD28 co-stimulation signal.

Regulation of NF-AT activation by the cAMP/PKA pathway in CD3/CD28 stimulation

Having observed the up-regulation of IL-4 gene expression by cAMP/PKA activation, we next studied the downstream signaling of the cAMP/PKA pathway and CD3/CD28. For this purpose, luciferase constructs that detect the activation of NF-AT, NF-κB, CREB, ERK, JNK and p38 signaling pathways were transfected in Jurkat-CD28 cells and then stimulated with anti-CD3 mAb alone or with anti-CD3/CD28 mAb in the presence of db-cAMP. As shown in Fig. 6(A), the stimulation with anti-CD3 plus anti-CD28 mAb increased the transcription

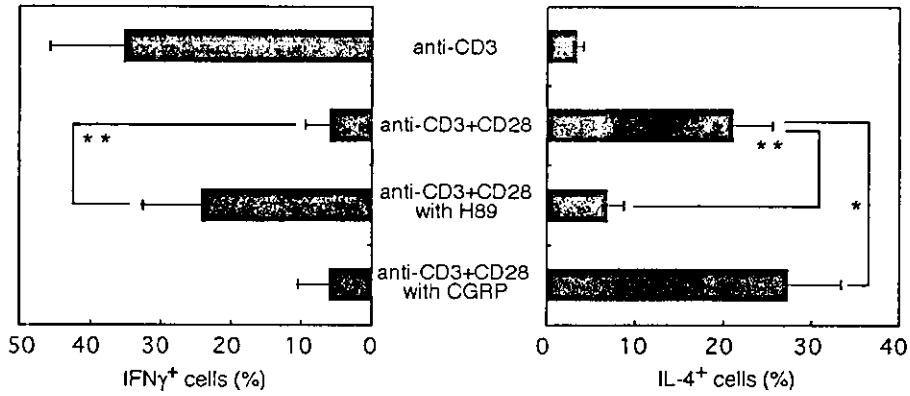

Fig. 5. Effects of H-89 or CGRP on T_h1/T_h2 polarization of $CD4^+$ T cells. $CD4^+$ T cells (10^6 cells/ml) were stimulated with 10 μg/ml anti-CD3 mAb and 1 μg/ml anti-CD28 mAb in the presence of either 20 μM H-89 or 1 nM CGRP. After 7 days, the primed $CD4^+$ T cells were re-stimulated with anti-CD3 mAb for 4 h in the presence of 2 μM monensin, and stained for intracellular IFN-γ and IL-4. Values are means ± SD (n = 6–7). $^*P < 0.05$, $^{**}P < 0.01$.

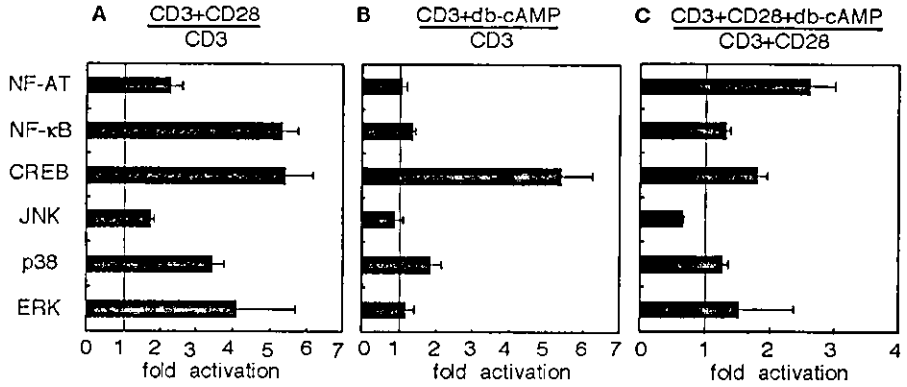

Fig. 6. Effects of db-cAMP on signal transduction of Jurkat-CD28 cells by CD3/CD28 co-stimulation. Aliquots of 5 μg $p(Ig\kappa)_3$-luc (NF-κB), pNF-AT72-luc (NF-AT) or pFR-luc plus 0.5 μg pFA-CREB (CREB), pFA-Elk (ERK), pFA-c-jun (JNK) or pFA-CHOP (p38) were transfected into Jurkat-CD28 cells. The cells were stimulated with 0.1 μg/ml anti-CD3 mAb and 1 μg/ml anti-CD28 mAb in the presence or absence of 5 μM db-cAMP for 16 h. The relative luciferase activity was measured with lumiphotometer. The results were expressed as fold activation of CD3/CD28 co-stimulation to CD3 stimulation (A), CD3 plus db-cAMP stimulation to CD3 stimulation (B) and CD3/CD28 co-stimulation plus db-cAMP to CD3/CD28 co-stimulation (C) (means ± SD, n = 3–4).

activity of all signaling molecules examined at ~2- to 5-fold the rate of that obtained by stimulation with anti-CD3 alone. When db-cAMP was added to Jurkat-CD28 stimulated with anti-CD3 mAb, a marked increase in CREB transcription activity was observed (Fig. 6B). This effect would be due to activation by direct phosphorylation of CREB by PKA. Interestingly, when Jurkat-CD28 was stimulated with anti-CD3 plus anti-CD28 mAb in the presence of db-cAMP, luciferase activities of CREB and NF-AT were markedly increased in comparison to those observed in the absence of db-cAMP (Fig. 6C).

To further examine whether the enhanced NF-AT activity was contributed to the activation of PKA pathway, this pathway was blocked with H-89 and expression of dominant-negative PKA (PKA-R^{G324D}) in the NF-AT luciferase assay. As shown in Fig. 7(A), H-89 significantly inhibited NF-AT transcription activity induced by CD3/CD28 co-stimulation, but not with CD3 stimulation alone. Similarly, forced expression of PKA-R^{G324D} also significantly inhibited NF-AT transcription activity activated by CD3/CD28 stimulation (Fig. 7B). Moreover, to address whether the cAMP/PKA pathway regulates NF-AT activation in not only Jurkat cell lines, but also naive $CD4^+$ T cells, a gel shift assay was performed (Fig. 7C). NF-AT-binding activity on CD3/CD28 stimulation was enhanced by CGRP, a physiological activator of the cAMP/PKA pathway, and strongly inhibited by H-89, an inhibitor of the cAMP/PKA pathway. These results indicated that CD3/CD28 stimulation induces the activation of the cAMP/PKA pathway resulting in increased NF-AT transcription activity and that a moderate activator of the cAMP/PKA pathway in the presence of a CD3/CD28 co-stimulation signal is able to further enhance NF-AT activity to a much greater extent.

Discussion

The present study demonstrated that the cAMP/PKA signal transduction pathway is critical for commitment to T_h2 differentiation via engagement of CD3 and CD28. Moreover, the cAMP/PKA pathway is further activated by CGRP in the

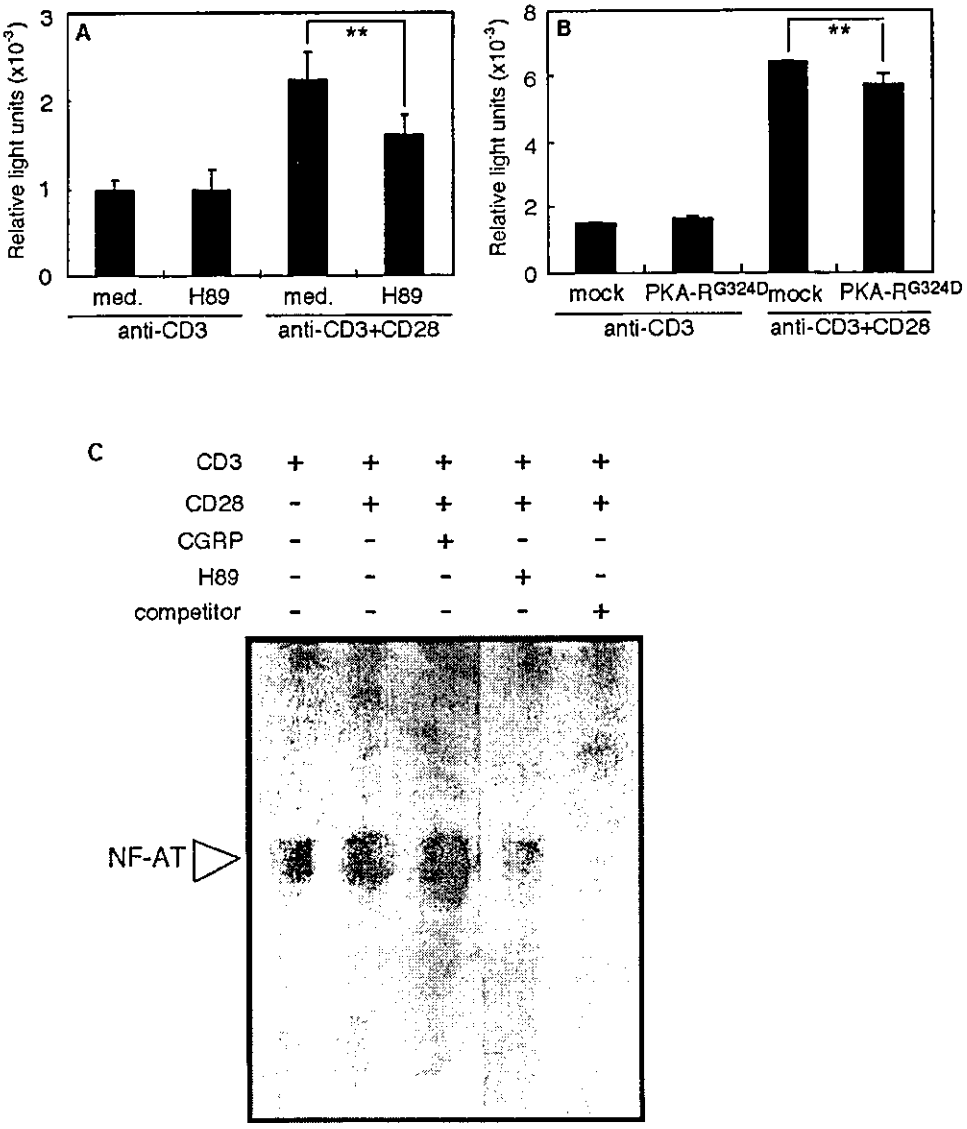

Fig. 7. PKA regulated NF-AT activity stimulated with anti-CD3 plus anti-CD28 mAb. (A) An aliquot of 5 μg pNF-AT72-luc (NF-AT) was transfected into Jurkat-CD28 cells. The cells were stimulated with 0.1 μg/ml anti-CD3 mAb (OKT3) and 1 μg/ml anti-CD28 mAb in the presence or absence of 20 μM H-89 for 16 h. (B) An aliquot of 5 μg pNF-AT72-luc was co-transfected with 15 μg $pCMV_T$-TK neo vector (mock) or $pCMV_T$-TK neo PKA-R^{G324D} (PKA-R^{G324D}) into Jurkat-CD28 cells. The cells were stimulated with 0.1 μg/ml anti-CD3 mAb and 1 μg/ml anti-CD28 mAb. Following the stimulation, the emitted light was expressed as relative light units. The data represents means ± SD (n = 4). $^{**}P < 0.01$. (C) Gel shift assay with NF-AT oligonucleotides as a probe. Naive CD4⁺ T cells were stimulated with anti-CD3 mAb or anti-CD3/anti-CD28 mAb in the presence or absence of CGRP or H-89 for 6 h and nuclear extracts were purified. An aliquot of 5 μg of protein was analyzed in a gel shift assay. Arrowhead indicates specific bands that bound to the NF-AT oligonucleotide and competition with 66-fold excess of unlabeled NF-AT oligonucleotide shows the specificity of the NF-AT interaction.

presence of a CD3/CD28 co-stimulation signal; this process thereby induces the up-regulation of IL-4 production, and the down-regulation of both IL-2 and IFN-γ production from mouse naive CD4⁺ T cells, resulting in the promotion of T_h2 differentiation in the immune system.

cAMP accumulation by CGRP has been reported in certain types of cells, including immune cells such as thymocytes (30), splenocytes (31) and T_h clones (32). Recently, the CGRP receptor was shown to be composed of CRLR and RAMP1 (33). CRLR is a heptahelical receptor coupled with $G_s\alpha$ and binding of CGRP to its receptors activates adenylyl cyclase (19). Since we have detected the expression of both CRLR and RAMP1 mRNAs in mouse CD4⁺ T cells (unpublished data), CGRP would induce cAMP elevation and PKA activation by a direct effect via its receptors on CD4⁺ T cells. In contrast, CD3 and CD28 molecules do not directly couple to adenylyl cyclase, although stimulation of anti-CD3 plus anti-CD28 mAb increased cAMP levels and PKA activity in mouse naive T_h cells. Therefore, CD3/CD28 would possess a signal transduction mechanism that induces indirect activation of adenylyl cyclase. One possible candidate of the activator is phospholipase C (PLC) γ1. CD3/CD28 engagement provokes rapid

increases in both tyrosine phosphorylation and the catalytic activity of PLCγ1 (34,35). The activated PLCγ1 hydrolyzes phosphatidylinositol-4,5-bisphosphate to inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 and DAG stimulate the release of Ca^{2+} from intracellular stores and activate protein kinase C (PKC) respectively. The increased intracellular free Ca^{2+} binds to calmodulin and the Ca^{2+}/calmodulin complex activates adenylyl cyclase (36). PKC also activates adenylyl cyclase catalysts by phosphorylation (37,38). Therefore, the cross-linking of CD3 and CD28 would indirectly activate adenylyl cyclase via the PLCγ1-mediated signaling pathway in T cells.

Studies on the effects of cAMP/PKA pathway on cytokine production from T cells have reported conflicting results. The consensus is that cAMP and its inducers suppress both T cell proliferation and the expression of T_h1 cytokine genes. We also confirmed the inhibition of IL-2 and IFN-γ production and T cell proliferation of CD3- or CD3/CD28-stimulated naive T_h cells by CGRP or db-cAMP. In contrast, the function of the cAMP/PKA pathway as regards production of T_h2 cytokines appears to be affected in different ways, depending on cell type, the method of cell stimulation and the concentration of cAMP or its inducers. Cholera toxin, which ribosylates the α subunit of the G stimulatory protein, leads to an accumulation of cAMP, and cAMP-elevating agents have no effect on IL-4 production and proliferation of T_h clones (39,40). On the other hand, it was reported that a high concentration of db-cAMP (1 mM) suppresses both mRNA accumulation and secretion of IL-4 from human T cells stimulated with anti-CD3 plus anti-CD28 antibodies (41). Another report demonstrated that cAMP up-regulates IL-4 and IL-5 production from $CD4^+$ T cells (42). In that study, mouse $CD4^+$ T cells were stimulated with concanavalin A, IL-2 and irradiated splenocytes for 3 days, and then re-stimulated with phorbol 12-myristate 13-acetate and ionomycin or with anti-CD3 mAb. The conclusion of that particular study was that, in activated $CD4^+$ T cells, cAMP induces a switch of lymphokine production towards a T_h2-like phenotype through regulation at the transcriptional level. However, the effect of cAMP in primary naive $CD4^+$ T cells stimulated with anti-CD3/CD28 antibodies has not been studied to date. We clarified for the first time that CGRP (in a concentration that induced ~2-fold increase in the level of cAMP, compared to that of the control) and db-cAMP (at a concentration <5 μM) reduced IL-2 and IFN-γ production, but augmented IL-4 production in anti-CD3/CD28 mAb-stimulated naive $CD4^+$ T cells.

As we have shown here, when compared to CD3 stimulation alone, CD3/CD28 co-stimulation up-regulated the transcription activities of NF-AT, NF-κB and CREB, as well as activated the ERK and p38 MAPK pathways in Jurkat-CD28 cells. The most interesting finding was that NF-AT transcription activity was markedly increased by db-cAMP in the presence of a CD3/CD28 co-stimulation signal. NF-AT is localized in the cytoplasm of resting T cells. TCR engagement activates the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin and calcineurin dephosphorylates NF-AT, allowing NF-AT to translocate from the cytoplasm to the nucleus (43–45). It has been reported that PKA suppress NF-AT translocation into the nucleus by phosphorylation (46). However, our study indicated that NF-AT transcription activity was activated by db-cAMP in the presence of CD3/CD28 co-stimulation; furthermore, the inhibition of PKA activity by the specific inhibitors suppressed NF-AT activity. From these results, we suggest that the cAMP/PKA pathway, activated by physiological stimulation and moderate concentrations of PKA activators, transduced a positive regulation signal enhancing NF-AT transcription activity. Recently, glycogen synthase kinase 3 (GSK3) was reported to regulate the nuclear export of NF-AT in T cells (47,48). Moreover, Fang *et al.* demonstrated that PKA could inhibit GSK3 activity by phosphorylation in some cell lines (49). Therefore, it is possible to infer that activated PKA might inhibit the nuclear export of NF-AT by inactivating GSK3 in $CD4^+$ T cells and that a longer presence of NF-AT in the nucleus might augment the transcription of T_h2 cytokine genes.

In our study, PKA inhibitor H-89 and dominant-negative PKA failed to inhibit NF-AT activation completely in CD3/CD28-stimulated T cells, whereas H-89 and PKI_{14-22} markedly inhibited IL-4 production and promoted T_h1 differentiation. These results suggest the existence of a transcription factor(s) other than NF-AT that is regulated by PKA for IL-4 production. We showed that CD3/CD28 co-stimulation induced a more significant activation of the p38 MAPK pathway and the CREB pathway than did CD3 stimulation alone. p38 MAPK is known to be an important integrator of TCR–CD28 stimulation of cell proliferation and cytokine production in primary naive T_h cells (50). Recently, it was reported that H-89 inhibited the phosphorylation of p38 MAPK by a cAMP-elevating agent, forskolin, whereas inhibitors of PKC, p70 (S6K) and phosphatidylinositol 3-kinase were ineffective in this regard (51). Moreover, Chen *et al.* showed that p38 MAPK is phosphorylated by the cAMP-dependent mechanism and that activated p38 MAPK phosphorylates GATA-3, resulting in promoting the production of both IL-5 and IL-13 in T_h2 cells (52). GATA-3 is a critical transcription factor of T_h2 cytokine gene expression, including the genes for IL-5, IL-13 as well as IL-4, via distal enhancers (53). It remains unclear whether or not PKA phosphorylates p38 MAPK in T_h0 cells; however, it is possible that the cAMP/PKA pathway activates p38 MAPK from our luciferase assays and cytokine array experiments. Moreover, our preliminary experiment showed an increase in GATA-3 mRNA by neuropeptide stimulation (unpublished data).

The signal transduction pathway responsible for CREB phosphorylation in T cells also remains unidentified. The candidates are Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV), which is highly expressed in T cells, and PKA. In studies using CaMKIV knockout mice, memory T cells showed reduced CREB phosphorylation and secreted less amounts of IL-2, IFN-γ and IL-4, compared to cells from wild-type mice, by stimulation of CD3/CD28 (54). These results suggest that CaMKIV-dependent CREB phosphorylation is important for the regulation of cytokine production in memory T cells. In contrast, naive T cells from CaMKIV-deficient mice are likely to normally produce cytokines and induce phosphorylation of CREB by CD3/CD28 co-stimulation, suggesting that PKA might regulate the phosphorylation of CREB in naive T cells.

In conclusion, the cAMP/PKA pathway transduces a critical signal for the regulation of IL-4 production of naive T_h cells stimulated with CD3/CD28. The signaling pathway modulates transcription factors such as NF-AT and CREB, and the

activation of the p38 MAPK pathway. The results of the present study emphasize the importance of the cAMP/PKA pathway for signal transduction in naive T_h cells stimulated with CD3/CD28.

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Abbreviations

AP-1	activated protein-1
APC	antigen-presenting cell
CaMKIV	calmodulin-dependent protein kinase IV
CGRP	calcitonin gene-related peptide
CREB	cAMP response element binding protein
CRLR	calcitonin receptor-like receptor
DAG	diacylglycerol
db-cAMP	dibutyryl-cAMP
ERK	extracellular signal-regulated kinase
GSK3	glycogen synthase kinase 3
H-89	*N*-[2-((*p*-bromocinnamyl)aminoethyl)]-5-isoquinoline-sulfonamide
JNK	c-Jun N-terminal kinase
IP_3	inositol-1,4,5-trisphosphate
MAPK	mitogen-activated protein kinase
OVA	ovalbumin
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
RAMP1	receptor activity modifying protein 1
TNF	tumor necrosis factor

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